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Polyoma Viruses with Mutations at Endonuclease *Hin*dII Site 1: Alterations at the COOH Terminus of VP1

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Four mutants of polyoma virus lacking endonuclease *Hin*dII site 1 were isolated and characterized with respect to the VP1 coding sequence. Three of these mutants had deletions that removed 0.2 to 0.3% of the genome. All three deletion mutants encoded VP1 proteins that were smaller than wild type and that lacked one or more tryptic peptides normally found in the wild-type VP1 protein. Our results suggest that *Hin*dII site 1 is at, or very near, the carboxy terminal end of the coding sequence for VP1. A model for the peptide organization in that region is presented.

Studies of thermosensitive and host range mutants have provided considerable insights into the biology and genetics of polyoma virus (6, 7). Most of these mutants, however, have been isolated after chemical mutagenesis and are likely to have multiple mutations. This has been demonstrated by a recent study of one mutant, *ts*59, in which the genes coding for viral late proteins contain multiple alterations, one of which renders the VP2/VP3 proteins thermolabile (13).

To help further define the role of viral gene products in cell transformation and to aid in the analysis of their biochemical properties, it is desirable to study viruses with single mutational alterations. Several laboratories studying simian virus 40 (SV40) biology have utilized viruses with limited nucleotide sequence alterations at specific sites (4, 5, 18, 24). Many of these mutants were constructed in vitro by treatment of restriction enzyme-generated linear DNAs with exonucleases; this often generates nonviable mutants with large deletions. To isolate viable mutants with single base changes or small deletions at specific sites, we have selected from unmutagenized virus stocks mutant DNAs that are spontaneously resistant to restriction endonucleases. Recently we described the isolation and preliminary characterization of mutants of polyoma virus lacking either or both of the endo *Hin*dIII sites (1a). In this report we describe four mutants lacking the endo *Hin*dII site near the junction of the 3′ termini of early and late mRNA's (1, 17, 28). Three of these mutants have small deletions and code for VP1 proteins that are smaller than wild-type VP1 as determined by polyacrylamide gel electrophoresis and two-dimensional peptide analysis.

MATERIALS AND METHODS

Cells and media. Secondary whole mouse embryo (WME) cells were used to prepare virus stocks and viral DNAs, and in plaque assays. Mouse 3T6 cells were used to prepare ^{14}C virus. BHK 21/13 cells were used for transformation assays.

The medium used in most experiments was a modification of Dulbecco minimum essential medium (MEM) similar to that described by Yamane et al. (31). Succinic acid (to 0.225 mM, pH 4.9) was added to Dulbecco MEM (GIBCO) before autoclaving. The medium was then cooled and supplemented with $NaHCO_3$ (1.5 mg/ml), tryptose phosphate broth (5%, vol/vol), glutamine (0.6 mg/ml), penicillin (100 U/ml), streptomycin (40 μg/ml), nystatin (50 U/ml), and calf serum (10%, vol/vol). Specifically depleted Dulbecco MEM was used for labeling virus with ^{14}C-amino acids (NEC-445; New England Nuclear Corp.) as described elsewhere (12). For the preparation of virus stocks, and for plaque assays, media were supplemented with horse serum (5%, vol/vol) instead of calf serum. The medium used for plaque assays was AutoPow MEM (Flow Laboratories) containing agar (9 mg/ml; Difco Laboratories), horse serum (3%, vol/vol), and dexamethasone (1 μM) (23). The low-phosphate modification of Dulbecco MEM used in preparing [^{32}P]DNAs has been described (10).

Virus stocks and DNAs. In this study, the Pasadena large-plaque polyoma virus was employed. The procedures used for preparing virus stocks, plaque assays, and DNA infections have been described (8, 10). Plaque-purified virus stocks were prepared by infection of WME cells with virus from well-isolated single plaques. Viral DNAs were purified by a modified Hirt procedure (1a).

Transformation of BHK 21/13 cells. The capacity of polyoma viruses to transform BHK 21/13 cells

was determined by plating infected cells in media containing 0.33% agarose, 10 μg of dextran sulfate per ml, 10% calf serum (but no tryptose phosphate broth) (21, 22). After 21 to 28 days at 37°C, colonies of growing cells were scored as transformed.

Endonucleases. Endonucleases *Hin*dIII, *Eco*RI, *Hpa*II, and *Bam*HI were prepared by modifications of previously published procedures (3, 25, 27, 29). Endo *Hha*I was purchased from New England Bio Labs.

Electrophoresis of DNAs. Agarose gels (16 by 12 by 0.3 cm) contained 1% agarose (SeaKem) in electrophoresis buffer (40 mM Tris-hydrochloride, 8.5 mM sodium acetate, 1 mM EDTA, pH 7.8). Samples were subjected to electrophoresis for 6 h at 40 mA/gel. Polyacrylamide gels (16 by 30 by 0.15 cm) contained 6% polyacrylamide and 0.5% bisacrylamide in electrophoresis buffer. Samples were subjected to electrophoresis for 8 to 12 h at 35 mA/gel. Analytical gels were dried under vacuum onto Whatman 3MM paper or Whatman DEAE paper and autoradiographed with Kodak RP-X-Omat film. The DNA was eluted from preparative gels by using a modification of the procedure of Wu et al. (30).

Electrophoresis of proteins and peptide analysis. The procedures used for electrophoretic separation of proteins in sodium dodecyl sulfate (SDS)-containing polyacrylamide gels and for two-dimensional separations of tryptic peptides have been described in detail elsewhere (12, 13).

Biochemical enrichment for DNAs lacking one endonuclease *Hin*dII site. Two separate isolation procedures were tried. In the first, a sample of polyoma DNA (prepared from cells infected with plaque-purified virus in such a way as to minimize defectives) was exhaustively digested with endo *Hin*dII, and the digestion products were separated by sedimentation through a neutral sucrose density gradient (10). The fractions likely to contain unit-length DNAs were pooled and dialyzed against Tris-saline buffer (8). WME cells were infected at 33°C with the DNAs and, after cytopathic effect developed, virus stocks were harvested. These stocks were used to prepare viral DNA which was exhaustively digested with endo *Hin*dII and the digestion products separated by electrophoresis through agarose gels. The ethidium bromide-stained DNAs corresponding to unit-length linear molecules were isolated and used to prepare virus stocks and, in turn, a third preparation of DNA. This DNA was digested with endo *Hin*dII, followed by electrophoresis through agarose gels to separate the digestion products. These DNAs were used to prepare virus stocks from which single plaques were isolated.

In the second isolation procedure, two different preparations of polyoma DNA were exhaustively digested with endo *Hin*dII, and the products were separated by electrophoresis. After staining with ethidium bromide, no band of unit-length linear DNAs was observed, but the area of the gel to which such molecules would be expected to migrate was excised, and the DNA was extracted. Virus stocks were prepared with this DNA and used to prepare more viral DNA, which was carried through another digestion and electrophoresis. This time, DNAs corresponding to unit-length genomes were visible. They were extracted from the gels and used to prepare virus stocks for single plaque isolations.

RESULTS

Isolation of polyoma viruses lacking an endo *Hin*dII site. Digestion of wild-type polyoma form I DNA by endo *Hin*dII yields two fragments of approximately 90 and 10% genome length (3, 9) (Fig. 1). If the viral DNA lacks one of the *Hin*dII sites, digestion of the form I DNA will yield only one fragment whose size will be nearly unit length if the DNA contains no large deletions. By using this difference as the basis for a selection technique, mutant viruses of essentially unit length that lack one of the *Hin*dII sites were isolated from unmutagenized Pasadena large-plaque polyoma virus.

Virus stocks obtained by infecting WME cells with DNAs carried through two or three enrichment steps with endo *Hin*dII (see above) were plaque assayed, individual well-isolated plaques were picked, and small stocks of virus were grown from each plaque. These plaque-purified viruses were then individually tested to determine whether they lacked a *Hin*dII site. Viral [^{32}P]DNA was prepared from each isolate and digested with endo *Hin*dII, and the digests were subjected to electrophoresis through agarose gels.

Four of the more than 200 plaque-purified virus isolates tested were identified as lacking a *Hin*dII site. Each of these four plaque isolates had only one species of form I DNA which was indistinguishable from that of wild-type polyoma form I DNA as determined by agarose gel electrophoresis. Digestion of these DNAs with endo *Eco*RI or endo *Hin*dII gave linear DNAs which comigrated (Fig. 2). Thus, the four mutants contain only one *Hin*dII site but have

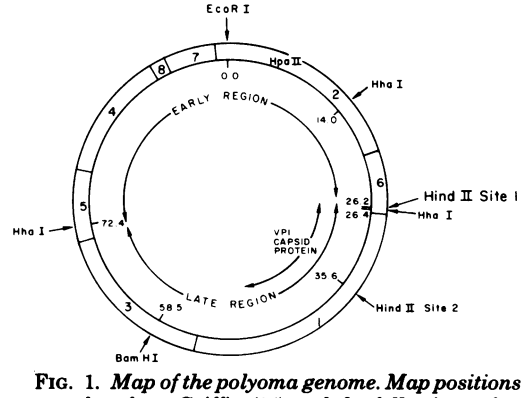

FIG. 1. *Map of the polyoma genome. Map positions were taken from Griffin (14) and the following references: endonucleases EcoRI and HpaII (16); endo BamIII (15); endo HindII (3, 9); VP1 capsid protein (26).*

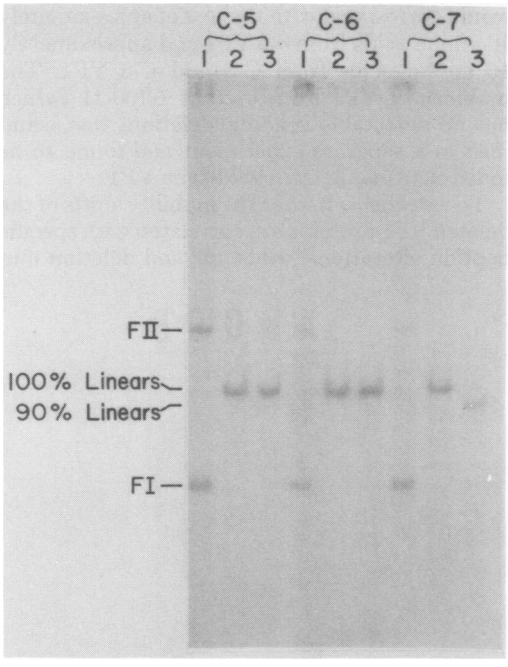

FIG. 2. *Autoradiogram of polyoma [^{32}P]DNA from isolates C-5, C-6, and C-7. For each isolate, slot 1 is an undigested sample of DNA, slot 2 is DNA digested with endo EcoRI (50 mM NaCl, 10 mM Tris, 10 mM $MgCl_2$), and slot 3 is DNA digested with endo HindII (10 mM NaCl, 10 mM Tris, 10 mM $MgCl_2$). Isolates C-5 and C-6 were identified as mutants lacking an endo HindII site. FI, Form I; FII, form II.*

retained most, if not all, of the remaining sequences in the polyoma genome.

Three of the four mutants were isolated from the first procedure described in Materials and Methods and cannot be considered to be independent isolates. However, as described below, physical characterization of their DNAs indicates that one isolate (mutant 6-5) differs from the other two (C-5 and C-6). The fourth mutant, 1-100-11, was derived from an independent sample of DNA used in the second procedure described in Materials and Methods and, based on its DNA structure and biochemical properties of its VP1 protein, this mutant differs from the other three (see below).

In addition to these four mutants, several other plaque-purified virus isolates containing mixtures of DNAs that lacked *Hind*II sites were identified in the initial selection. Many of these DNAs were substantially smaller than wild-type DNA and probably are defective genomes which require complementation by helper viruses. The biological properties of such defective genomes are difficult to study, and we have not pursued their characterization.

Characterization of the mutant DNAs. The four mutants described above were plaque-purified twice more, and three-times plaque-purified stocks were used in further characterization of the mutants. To determine which of the two *Hind*II sites each mutant lacked, samples of [^{32}P]DNA from each mutant were digested with mixtures of endo *Hind*II plus either endo *Eco*RI or *Bam*HI, and the products were separated by agarose gel electrophoresis. Digestion of each of the mutants with endonucleases *Hind*II plus *Eco*RI generated fragments of 0.64 and 0.36 genome length. Digestion with endonucleases *Hind*II plus *Bam*HI produced fragments of 0.77 and 0.23 genome length. These results indicate that all four mutants have retained *Hind*II site 2 but lack *Hind*II site 1 (Fig. 1).

Digestion of DNA preparations from each of the 4 mutants with endo *Hpa*II indicated that three of them (6-5, C-5 and C-6) have a slightly smaller *Hpa*II fragment 6 (*Hpa*II-6) than does wild-type DNA (Fig. 3). The size of *Hpa*II-6 from mutant 1-100-11 is indistinguishable from that of wild-type DNA. Since *Hind*II site 1 is located in *Hpa*II-6 (Fig. 1), it is likely that the apparent reduction in size of *Hpa*II-6 seen in mutants 6-5, C-5, and C-6 is due to deletions which include *Hind*II site 1. The sizes of the deletions have been estimated by measuring the mobilities of the altered *Hpa*II-6 fragments. Mutant 6-5 lacks approximately 0.2% of the genome (10 base pairs), and mutants C-5 and C-6 lack approximately 0.3% of the genome (15 base pairs). Mutant 1-100-11 has either no deletion or a deletion of less than five base pairs. All four mutants give the same endo *Hha*I digestion pattern as wild-type DNA, indicating that none of the deletions in these mutants include the nearby *Hha*I site which is within 20 base pairs of *Hind*II site 1 (14).

Characterization of the VP1 capsid protein of the mutants. Since the site of these deletions is close to the 3′ end of the 16S mRNA molecule that encodes polyoma VP1 (1, 17, 28), it was of interest to determine whether the genomic deletions have an effect on VP1. Cultures of 3T6 cells were infected with either wild-type polyoma virus or with mutant virus and radiolabeled from 48 to 60 h postinfection by growth in the presence of a mixture of ^{14}C-amino acids (2.5 μCi/ml). After radiolabeling, the infected cells were scraped from their culture dishes and separated into nuclear and cytoplasmic fractions by using Nonidet P-40. The proteins were solubilized and analyzed by electrophoresis through SDS-containing polyacrylamide gels, all as described elsewhere (12). Figure 4 shows a fluorograph of the proteins in the nuclear fraction after electrophoresis and demonstrates the following. First, the minor capsid proteins VP2 and

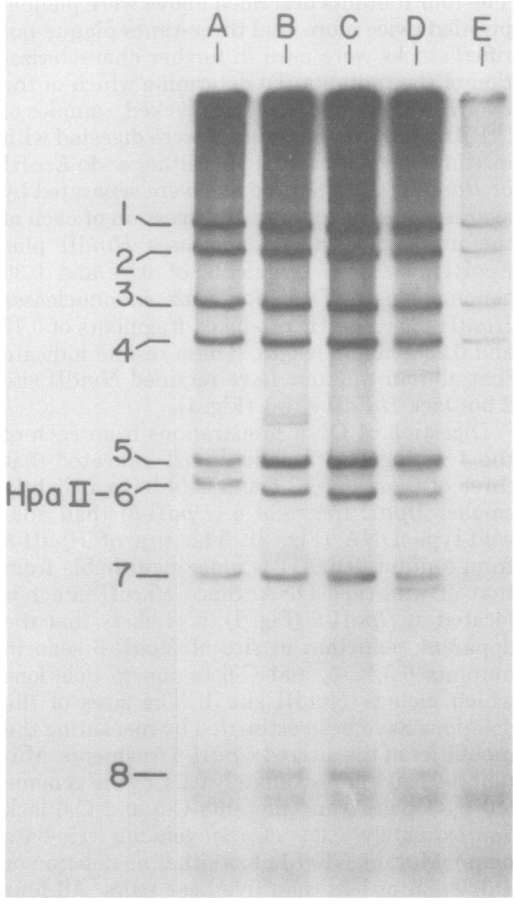

FIG. 3. *Autoradiogram of polyoma DNA digested with endo HpaII. Samples of [^{32}P]DNA from wild-type polyoma virus and from mutants 6-5, C-5, C-6, and 1-100-11 were digested with endo HpaII (10 mM Tris, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 6 mM KCl). The digests were separated by polyacrylamide gel electrophoresis. Minor bands in several of the tracks are due to defective DNAs which arose during the preparation of viral [^{32}P]DNA. Slot A is wild-type virus; slot B is mutant 6-5; slot C is mutant C-5; slot D is mutant C-6; slot E is mutant 1-100-11.*

VP3 of wild-type virus (B) are electrophoretically indistinguishable from their counterparts in mutants C-5 (C), C-6 (D), and 6-5 (E). Second, VP1, VP2, and VP3 of the wild-type strain used in these studies have the same apparent sizes as their electrophoretic counterparts from a previously characterized (13) wild-type strain (not shown). Third, the mobility of VP1 in the three deletion mutants is greater than that of wild-type VP1. Based on the molecular weights of 55,000 for tubulin, 45,000 for wild-type VP1, and 42,000 for actin, the mutant VP1 proteins were estimated to be 43,800 daltons (6-5) and 43,100 daltons (C-5 and C-6). These mobility shifts would correspond with the loss of approximately 10 amino acids from 6-5 VP1 and approximately 16 amino acids from C-5 and C-6 VP1. The mobility of VP1 from mutant 1-100-11 (which has no detectable genomic deletion) was examined in a separate experiment and found to be indistinguishable from wild-type VP1.

To establish whether the mobility shifts of the deleted VP1 proteins are correlated with specific peptide alterations, wild-type and deletion mu-

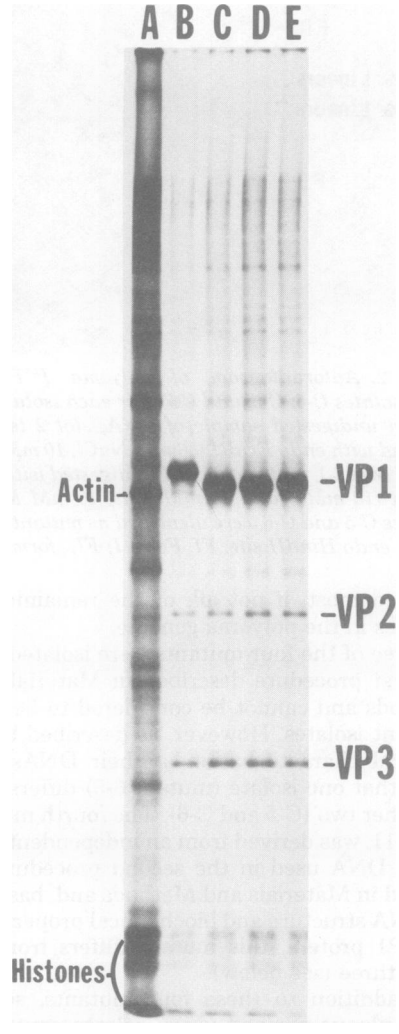

FIG. 4. *Fluorogram of intranuclear polyoma virus proteins. Nuclei recovered from infected cells grown in the presence of ^{14}C-amino acids were solubilized and subjected to electrophoresis in a 14% diallyltartardiamide-cross-linked polyacrylamide gel. Shown here is a photograph of a fluorogram prepared from the stained and processed gel. Nuclear fractions analyzed were obtained from uninfected cells (A), cells infected by wild-type virus (B), and cells infected by mutants C-5 (C), C-6 (D), and 6-5 (E).*

tant VP1 proteins were radiolabeled in vivo as described above and separated by electrophoresis in preparative polyacrylamide gels. After their recovery from the gels, the proteins were oxidized with performic acid and digested with tolysulfonyl phenylalanyl chloromethyl ketone-trypsin. The resulting tryptic peptides were subjected to two-dimensional separations on cellulose thin-layer plates and detected by autoradiography. The peptide distribution found for wild-type VP1 was essentially indistinguishable from that described previously (13). The only differences detected between the wild-type VP1 peptide distribution and those of the deletion mutants occurred in a cluster of spots designated "f–j" (Fig. 5). It is apparent that all three of the deletion mutant VP1 proteins are missing spot "f" (compare panels B-D with A), and that mutant 6-5 VP1 is missing spot "j" as well (panel C). As discussed below, it is suggested that the molecular weight difference between wild-type VP1 and mutant 6-5 VP1 (i.e., 1,200) reflects the loss of peptides "j" and "f" from the protein. Mutant C-5 and C-6 VP1 proteins are thought to have lost most if not all of peptide "f".

Thermolability of mutant viruses. To determine whether the altered VP1 proteins influenced the stability of the virion, samples of each mutant were incubated at 62°C. The extent of virus inactivation that resulted from the heating was determined by plaque assay of portions heated for up to 20 min. No significant difference was observed between the decay of infectivity of wild-type virus and that of any of the mutants (Fig. 6).

Biological characterization of the mutants. During the enrichment steps and in their isolation, viruses were grown at 33°C to avoid selecting against thermosensitive mutants. However, when tested, none of the four mutants exhibit altered growth properties at low (33°C) or high (37 and 39.5°C) temperatures, indicating that the alterations in VP1 have no effect upon virion assembly. Titers of mutant virus stocks prepared at 33°C were comparable to the titers of wild-type virus stocks prepared by using similar cells. A careful comparison of the growth curves of mutant 6-5 and wild-type virus indicates that there is no difference between the two (not shown). Each of the mutants, as well as the wild-type virus, grew more poorly at 39.5 than at 33°C, but the reduction in titer was the same for each of the mutants as for wild-type virus (Table 1). Also consistent with the conclusion that the mutations have no effect upon virion assembly are the equivalent efficiencies of plaque formation and the similarity in plaque morphology between the mutants and wild-type virus at either 33 or 37°C (not shown).

Three of the mutants lacking *Hind*II site 1 were tested for their ability to transform BHK 21/13 cells. The results from two sets of transformation assays are given in Table 2. Within

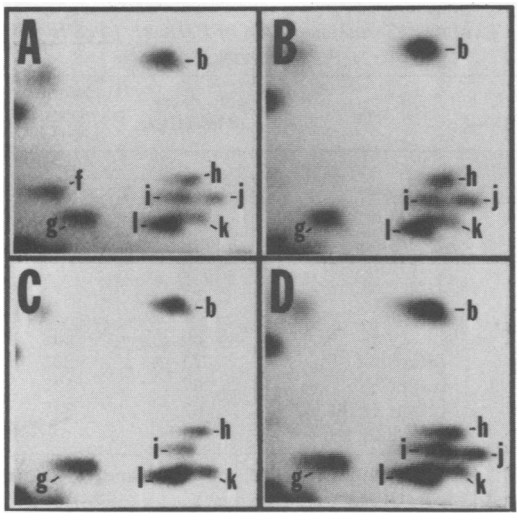

FIG. 5. *Tryptic peptide distribution of VP1 proteins from wild-type polyoma virus and mutants C-5, C-6, and 6-5. Tryptic hydrolysates prepared as described in the text were compared by two-dimensional separations on thin-layer cellulose plates. Shown here is a subset of VP1 peptides from wild-type (A) and mutants C-5 (B), 6-5 (C), and C-6 (D). For purposes of comparison, the letters used to identify specific peptides are the same as those used previously (13). Electrophoretic separation was from left to right, and subsequent chromatography was from bottom to top.*

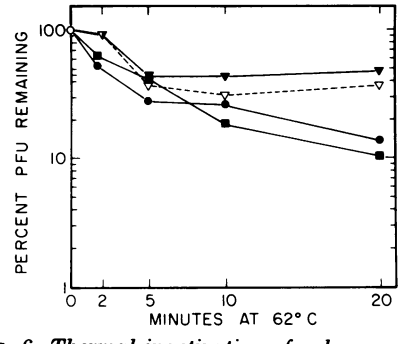

FIG. 6. *Thermal inactivation of polyoma viruses. Concentrated virus stocks were diluted to approximately 1 × 10^8 PFU/ml in Tris-saline buffer containing 500 μg of ε-aminocaproic acid. Tubes containing 0.5-ml aliquots were immersed in a 62°C water bath for 0, 2, 5, 10, or 20 min, then immediately immersed in an ice bath. All virus samples were then plaque assayed at 33°C. The open triangles indicate wild type. The filled symbols indicate mutants: ●, 6-5; ▲, C-5; ■, 1-100-11.*

TABLE 1. *Growth properties of polyoma viruses*[a]

Virus	Yield (PFU/ml) at:		Yield (%)[b]
	33°C	39.5°C	
Wild type	4.7×10^7	6.7×10^6	14
Mutant 6-5	1.5×10^8	1.4×10^7	9
Mutant C-5	1.6×10^8	2.0×10^7	13
Mutant C-6	1.0×10^8	2.1×10^7	21

[a] Two 60-mm plates of WME cells were infected with each virus stock at a multiplicity of approximately 1 PFU/cell. One of the plates was placed at 33°C, and the other was at 39.5°C. After a 1-h adsorption period, prewarmed medium was added to each plate. The plates were incubated for 6 days at either 33 or 39.5°C. Virus was then harvested by scraping the cells into the medium. The virus stocks were frozen and thawed three times and plaque assayed at 33°C.

[b] Values represent yield at 39.5°C/yield at 33°C.

the limits of quantitation possible in such an assay, the mutants appear to transform as efficiently as wild-type polyoma virus.

DISCUSSION

We can only speculate about the origin of the four mutants that have been isolated. Although our selection procedure must enrich for mutants lacking *Hin*dII sites, it may also generate them. As endo *Hin*dII generates blunt-ended DNAs (11, 20), recircularization of *Hin*dII linears is likely to occur within the cell by blunt-end ligation or by intramolecular recombination. Three of the mutants we have isolated contain demonstrable deletions; they may have acquired the deletions by a process of intramolecular recombination similar to that suggested by Lai and Nathans (19). Alternatively, their deletions may have occurred spontaneously before our selection. The fourth mutant exhibits no detectable deletion. If its resistance to endo *Hin*dII is due to a single base change, it is not likely to be derived from a recombinational event at *Hin*dII site 1. More likely, it is the result of a mutation that was spontaneous or caused by blunt-end ligation.

Hybridization studies with polyoma late mRNA's (1, 17, 28) and biochemical studies of the late mutant *ts*59 (13) have suggested that the coding sequence for the carboxy-terminal end of polyoma VP1 extends at least to 26 map units. Our results demonstrate that deletions at *Hin*dII site 1 in polyoma DNA (at 26.2 map units) reduce the size of VP1 but, remarkably, do not affect the stability of the polyoma virions. Analogous mutants of SV40 have been obtained where deletions near the nucleotide sequence coding for the carboxy-terminal end of SV40 VP1 result in viable mutants with VP1 capsid proteins that are apparently 1,000 to 4,000 daltons smaller than SV40 wild-type VP1 (4). These SV40 mutants, however, unlike the polyoma mutants, grow more slowly than wild-type virus.

In all three polyoma deletion mutants, VP1 tryptic peptide "f" was consistently missing (Fig. 5). The simplest explanation of this result is that the DNA sequence at *Hin*dII site 1 encodes VP1 peptide "f" which includes, or is near, the carboxy terminus of the protein. Figure 7 proposes a model for the peptide organization which is compatible with our results. Since all three deletions remove the *Hin*dII site at 26.2 map units, they must overlap at that locus. In addition, since the *Hha*I site at 26.4 map units is unaffected, none of the deletions extends to include that site. VP1 peptide "f" is shown to be closer to the carboxy terminus of VP1 than peptide "j"

TABLE 2. *Transformation of BHK 21/13 cells by polyoma viruses*

Cell type	Multiplicity of infection	Transformed colonies/cells infected[a]
Expt 1		
Wild type	82	176
Mutant 6-5	109	90
Mutant C-5	100	125
Mock-infected	0	0
Expt 2		
Wild type	80	23
Mutant 6-5	73	26
Mutant C-5	67	13
Mutant 1-100-11	87	33
Mock-infected	0	0

[a] In experiments 1 and 2, 4.4×10^5 and 6.0×10^5 cells, respectively, were infected.

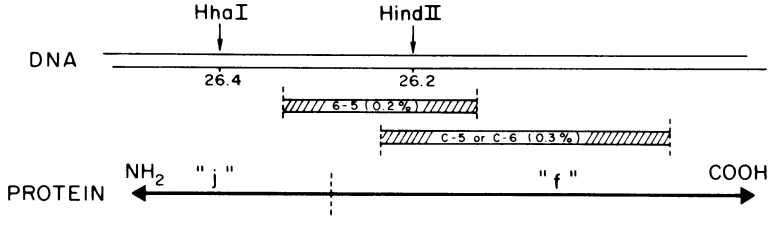

FIG. 7. *Hypothetical model for the organization of VP1 peptides around HindII cleavage site 1.*

because it is absent in the VP1 proteins of all three mutants. If the order were reversed, mutants C-5 and C-6 would retain peptide "j" only by a precise, in-frame internal deletion of the coding sequence for peptide "f". These two peptides have previously been positioned, but not ordered, near the carboxy-terminal end of VP1 by independent procedures (13).

The sizes of the VP1 proteins of these mutants as estimated from SDS gels are somewhat smaller than expected from our estimates of the sizes of their genomic deletions. An explanation of this discrepancy could be that proteolysis or premature chain termination may additionally shorten the VP1 protein. Both types of gel analyses are subject to error, and this may contribute to the discrepancy.

A second observation which is not easily explained is that the VP1 of mutant 6-5 which is missing both peptides "f" and "j" migrates in the SDS gel system as if it is larger than the VP1 proteins of mutants C-5 and C-6 which are missing only peptide "f." One explanation of this apparent inconsistency is that the electrophoretic mobility of polyoma VP1 is influenced by charge or conformational properties conferred on the molecule by amino acids at the extreme carboxy-terminal end. This observation could also be accounted for if peptides "f" and "j" or "f" and a fragment of "j" were fused to form a new peptide such as was observed with the VP1 protein of mutant *ts*59 (13). However, since no new peptides were detected, and since there were no apparent quantitative increases in other spots (as would be expected if the new peptide comigrated with another spot), this possibility seems unlikely.

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